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Non-invasive genetic tracking of Harbor Seals (Phoca vitulina)

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Non-invasive genetic tracking of Harbor Seals (Phoca vitulina)

By

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Accepted in Partial Completion
Of the Requirements for the Degree
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MASTER'S THESIS

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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
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March 2015

Abstract

Understanding the effect of individual differences on trophic interactions of upper-level predators, which can have disproportionate effects on an ecosystem, is imperative for successful management of populations. Marine mammals that prey on fish species of commercial and conservation importance are thus of particular interest. However, quantitatively monitoring and evaluating the impact of marine mammals on the environment is challenging because it is difficult to observe, capture, and collect repeated samples of individuals. Molecular genetic analysis of scat provides an inexpensive and feasible option to address these challenges. I developed an innovative non-invasive method for re-sampling individual marine mammals by collecting harbor seal (*Phoca vitulin*a) scat at a haul-out in Cowichan Bay, B.C. I chose to study this species because it is the most abundant pinniped in the inland waters of the Pacific Northwest and a notable predator on fisheries stocks. In addition, a Python-based computer program for experimental design, incorporating genotyping error, was created to determine the sampling schemes needed to genetically track individuals of any taxa with site fidelity. My results demonstrate that non-invasive individual tracking via microsatellites can be successfully implemented in marine mammals. Furthermore, the optimum sampling scheme to track individuals over a given time frame at the study site requires 690 samples over 23 bouts (30 samples per bout). These genetic-tracking and sampling scheme methodologies can be applied to help answer several biological questions including diet, relatedness, population structure and impacts on species of interest.

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Table of Contents

List of Figures

List of Tables

List of Appendices

Introduction

Data that track individuals in the wild are fundamental to answering broad questions such as those relating to population structure, trophic interactions, behavioral patterns, and life history events (Clutton-Brock & Sheldon 2010). Especially with top predators that can have drastic effects on an ecosystem, understanding their dynamic roles and individual variation is of prime importance (Myers *et al.* 2007; Heithaus *et al.* 2008). Individual-based data sets can elucidate intraspecific differences in areas such as trophic and foraging ecology (Newsome *et al.* 2009; Arnould *et al.* 2011; Hückstädt *et al.* 2012), population dynamics (Vindenes *et al.* 2008), and disease ecology (Johnson *et al.* 2009), that may highlight important patterns and processes dictating interactions among species (Bolnick *et al.* 2003; Cianciaruso *et al.* 2009). Individual variation in ecological traits has important implications because it can create variance in demographic parameters (Bolnick *et al.* 2011). For example, in a study showing evidence of diet specialization in California sea otters (*Enhydra lutris nereis*), the use of resources by different individuals affects the demographics of energetic needs and habitat choice within the population (Estes *et al.* 2003). Individual variation in diet can also be an important source of data for understanding broader processes such as food-web interactions and foraging strategies (Estes *et al.* 2003; Svanbäck & Persson 2004). Although there are many examples of individual variation in a variety of taxa (see reveiw Bolnick *et al.* 2003), investigations as to how traits, such as diet, habitat selection, or foraging behavior, are distributed among individuals are still relatively unknown (Araújo *et al.* 2010). Given this gap in knowledge recent studies have examined individual diet specialization in species such as southern elephant seals (*Mirounga leonina*) (Hückstädt *et al.* 2012), California sea otters (Estes *et al.* 2003), seabirds (Woo *et al.* 2008), and many other taxa (Pires *et al.* 2011). In particular for marine systems, studies investigating diet and foraging strategies are of increasing importance as many of the predator-prey interactions involve species of conservation and commercial interest (Williams *et al.* 2011; Bowen & Lidgard 2013). A possible method for researchers to investigate individual differences in trophic interactions is through longitudinal studies of individuals, which observe repeated instances of a niche trait, such as diet (Bolnick *et al.* 2002). Yet, previous studies have not incorporated a method to efficiently track individuals and are subjected to labor intensive field observations

(Newsome *et al.* 2009)or through invasive capturing of individuals (Hückstädt *et al.* 2012), all of which can limit recaptures of individuals.

Harbor seals (*Phoca vitulina*) highlight the importance of tracking individual variation in diet. These upper-trophic level marine mammals are the most abundant pinniped species in the inland waters of the Pacific Northwest (Jeffries 2000) and a notable predator on fisheries stocks (Olesiuk 1993). Despite their significant role in the ecosystem, we know little about the trophic interactions of harbor seals due to the difficulty in studying them (Harwood 1983; Gulland 1987; Bowen 1997; Morissette *et al.* 2012). While harbor seals are typically regarded as generalist predators, their populations may actually be comprised of individuals with specialized diets (Lance *et al.* 2012; Bromaghin *et al.* 2013; Bjorland *et al.* Accepted). This potential for specialization can have ramifications for understanding harbor seal influence on fish stocks and makes it necessary to develop a method to track individuals in the system. However, previous approaches to collecting individualized data on marine mammals have usually required conducting expensive, invasive, and impractical manipulation experiments (Williams *et al.* 2004; Read 2008), such as stomach contents (Jansen *et al.* 2013) and tissue biopsies for fatty acid (Andersen *et al.* 2004) or stable isotope (Arnould *et al.* 2011) analyses. The invasiveness of these methods leave researchers subjected to increased logistical concerns in capturing and handling individual animals and therefore limit the number of recaptures; an integral facet of longitudinal studies (Johnson 2002). Given these methodological drawbacks, it has been difficult to study and track individual marine mammals effectively (Bowen 1997; Williams *et al.* 2004; Morissette *et al.* 2012).

Non-invasive genetics methods in wildlife conservation and management provide a solution to the logistic concerns of studying individual variability in marine mammals. Non-invasive genetics can be defined as gathering data without handling, capturing, or continuously observing a target species. Due to the accelerated rate at which molecular methods have been developed, the accessibility and costs associated with these techniques have become a realistic option for biologists and provide a quantitative approach for individual and population monitoring (DeYoung & Honeycutt 2005; Waits & Paetkau 2005). Specifically the affordability of techniques such as analyses of microsatellites—which are highly polymorphic markers among individuals—are

instrumental in addressing genetic drift, genetic variation, and relatedness within a target population (Selkoe & Toonen 2006; Ouborg *et al.* 2010; Guichoux *et al.* 2011). Non-invasive genetic sampling has been applied to answer a variety of ecological questions, such as identifying the presence of rare or elusive species (Foote *et al.* 2012), determining gender through sex-linked chromosomes (Reed *et al.* 1997), identifying diet items (Deagle *et al.* 2005; Deagle *et al.* 2007), and evaluating genetic diversity, population structure, and mating systems (Palsbøll *et al.* 1997; Garnier *et al.* 2001). Non-invasive genetic sampling methods are able to obtain DNA samples from a variety of sources such as hair, feces, urine, skin, feathers, egg shells, and saliva. Each sample from these sources contains genomic DNA (gDNA)(Waits & Paetkau 2005). Due to their behavior of hauling-out on land, harbor seals afford an opportunity to obtain DNA from scat. Hauling-out is a behavior commonly associated with pinnipeds that allows for periods of rest between foraging activities (Hoelzel & editors 2009, p 197). This resting behavior is advantageous for obtaining samples non-invasively; a method previously employed for individual identification (Reed *et al.* 1997) but yet to be applied for tracking individuals. While obtaining scats from haul outs can be deemed a harassment of harbor seals, this is a less invasive means to collect genetic samples than through blood or tissue biopsies. Historically, conventional tags tracking individuals have been human-made through colored bands or brands, or through individual morphological marks on the animal and using photo identification. More recently however, there has been potential to use a "permanent" genetic tag to circumvent the need to capture animals or when there is little phenotypic differences among individuals. A genetic tag fulfills many important characteristics necessary to track individuals effectively, including universal applicability, non-invasiveness, no significant loss of tags, lack of ambiguity among individuals, and rapid matching of tags once established (Palsbøll 1999). Considering the likelihood of obtaining non-invasive samples from harbor seals through scat this species offers a suitable system to develop a method to track individuals.

In the study of marine mammals non-invasive genetic sampling has been a promising technique employed to address ecological and evolutionary biological questions in different taxa such as Atlantic spotted dolphins (*Stenella frontalisis*) (Green *et al.* 2007), bottlenose dolphins (*Tursiops truncatus*) (Parsons 2001), killer whales (*Orcinus orca*) (Ayres *et al.* 2012), grey seals (*Halichoeru grypus*), harbor seals (Reed *et al.* 1997), and ringed

seals (*Phoca hispida*) (Swanson *et al.* 2006). However, a method for tracking marine mammals genetically has only been established in humpback whales (*Megaptera novaeangliae*) (Palsbøll *et al.* 1997). The lack of generic methods to genetically track individuals stems in large part from a necessity to use species-specific genetic markers (Selkoe & Toonen 2006) . My study applied the available library of microsatellite markers to harbor seals previously developed only from tissue or blood samples and apply these markers to scat samples. In addition to the need for developing species specific genetic markers, longitudinal non-invasive genetic tracking comes with a set of challenges that includes sampling logistics (number of samples needed to track multiple individuals), genotyping error associated with lab methods, and their combined effect in developing an efficient non-invasive genetic project.

Major considerations regarding non-invasive genetic tracking include logistics and costs. Both sampling (number of samples/bouts) and genotyping (lab work/genotyping error) necessary to track individuals force researchers to make trade-offs in the design of their project (Hoban 2014). I define sampling design as the number of samples, bouts, and genetic markers used to appropriately address a research question. Sampling design has been previously highlighted as an important component to improving accuracy in non-invasive population studies (Marucco *et al.* 2011). However, most of the number of bouts and therein the number of samples collected for previous non-invasive studies were completed *a posteriori* because there were no defined sampling schemes and therefore researchers were subjected to continuous sampling (Lukacs & Burnham 2005; Marucco *et al.* 2011). Few exceptions have specifically mentioned sampling schemes for non-invasive genetics (Solberg *et al.* 2006; Marucco *et al.* 2012), and these studies were specific for population-based questions, such as population size estimation via non-invasive recaptures, which require fewer re-samples than tracking individuals to determine estimates.

In addition to sampling design, genotyping errors in identifying individuals are usually taxa-and sample quality specific and thus methodological generalizations can present serious challenges (Taberlet & Luikart 1999). Genotyping error is associated with allelic dropout (homozygote for a locus when a sample should be heterozygote) and false alleles (an incorrect allele identified due to artefacts from PCR) (Pompanon *et al.* 2005). One major concern that can dictate error rates is the amount of quality DNA available when using hairs,

feathers, or feces from animals. Due to the indirect nature of the samples (as opposed to tissue or blood samples) the DNA within the samples is usually more degraded. Degraded DNA may lead to increased error rates when genetically identifying individuals (Taberlet & Luikart 1999). Studies have used a variety of ways to minimize these errors, such as repeatable PCR experiments to confidently genotype each individual (Taberlet *et al.* 1996; Garnier *et al.* 2001) and assessing these repeat PCRs through programs such as GIMLET (Valière 2002) or GEMINI (Valière *et al.* 2002). Conversely there are programs that use a statistically conservative approach route to minimize error, such as CERVUS (Kalinowski *et al.* 2007), PEDANT (Johnson & Haydon 2007) and DROPOUT (McKelvey & Schwartz 2005; Schwartz *et al.* 2006). This component to experimental design is vital to successfully identifying individuals, although all but PEDANT require potentially cost-prohibitive repeated PCRs. PEDANT is the lone option for applying data with no reference genotypes. By using a maximum-likelihood algorithm the program is an advantage in studies that may lack resources to perform repeat PCRs on the entire data set (Johnson & Haydon 2007). However PEDANT does not distinguish individuals as it is solely for estimating error. Developing a successful non-invasive genetic study requires considering both genotyping error and sampling design.

With number of samples and genotyping error being a critical component this study attempted to combine these factors through an efficient computer modelling program. There are few tools available to researchers to define parameters *a priori* for non-invasive genetic tracking. GEMINI (Valière *et al.* 2002), the only tool available for study design, is only applicable for repeated-PCR experiments and have yet to combine more cost efficient error estimates (as shown with the program PEDANT) with experimental design. To date, no one has examined the study-design requirements for individual tracking to answer broader questions such as diet, resource use, or disease ecology. I proposes a hybrid approach that integrates both repeated PCRs and a computer-based approach for addressing genotyping error when matching individual samples.

Presented with a suitable system in harbor seals to track individuals and a lack of tools for researcher to appropriately design individual-based studies, I report the results of a study that developed an innovative non-invasive method to genetically track individual harbor seals. By collecting harbor seal scat at a haul-out in Cowichan Bay, British Columbia, I successfully genotyped and sexed fecal samples using nine microsatellite

loci and *ZFX/ZFY* qPCR gender determination. In addition, this study developed a Python-based experimental design model that simulates non-invasive genetic tracking and genotyping error by incorporating PEDANT specifically for individual-based ecological questions, thus assisting researchers as they develop future projects. The Python model is available at: https://github.com/McGlock/WisePair. My goals were to develop a method to genetically track individual harbor seals and to develop an *a priori* optimal sampling scheme to genetically track individuals of any taxa.

Methods

Sampling and DNA extraction

I collected 46 scat samples from harbor seals in three sampling periods during January – March 2014 (Jan. n=21, Feb. n = 12, Mar. n=13) from a single haul-out site in Cowichan Bay, Vancouver Island, British Columbia (Figure 1). This site was an ideal candidate for the study due to its relative isolation in an estuarial bay and the relatively small (ca. 100 individuals) number of seals that actively use the site (Olesiuk 2009). The haul-out is comprised of floating logs (log booms) that are available to harbor seals year-round (Cottrell 1995; Baird 2001). During sampling trips, I opportunistically sampled harbor seal scats by fully surveying the log booms. Scats were selected based on subjective freshness, whereupon the level of moisture of the scat was indicative of having been recently deposited. Once a scat was identified as fresh, I swabbed approximately 75-100% of the exterior of the scats with a sterile cotton-tipped applicator to target the visible exterior mucus (Rutledge *et al.* 2009). After swabbing, cotton tips were stored in 2mL screw-cap vials with EtOH (95%) and at -20°C until gDNA extraction. Collection of samples was conducted with or by Sheena Majewiski, Research Biologist at the Department of Fisheries and Oceans Canada, under "Licence to Study Marine Mammals for Research Purposes MML-003".

Once samples were ready for DNA extraction, the excess EtOH was drained from the 2mL vials and the samples were dried in a drying oven at 60° C until all EtOH had evaporated. Due to the nature of swabbed samples, the majority of target DNA was epithelial cells and not scat matrix containing potential inhibitors. Therefore the use of a specialized stool extraction kit was deemed unnecessary. Instead, samples were extracted

using a standard Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, California). I quantified total gDNA (μg/μL) using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Delaware, USA) and verified samples readings ≤10 μg/μL using a Qubit® 2.0 Fluorometer (Life Technologies, Valencia, California). All samples ≥10 μg/μL were then diluted to DNA concentrations of 10 μg/μL.

Individual Genotyping

Based on an available published library of over 20 microsatellite markers for harbor seals (Burg 1996; Gemmell *et al.* 1997; Davis *et al.* 2002) I identified nine microsatellite markers that were developed from tissue to apply to our samples. To accomplish this identification I screened 18 potential markers; however, only, nine of them were positive for PCR amplification and used in this study: *LW20*, *HI15*, *TBPv2*, *M11*, *SGPv10*, *Lc5*, *Lc26*, *Pv11*, and *BG*. The other 9 markers that were tested but insufficient for scat genotyping were: *LW10*, *Lc6*, *HI16*, *Lc13*, *Pv3*, *Pv10*, *Hg6.1*, *Hg6.3*, *Hg8.9*, *SGPv11*, and *SGPv9*. The amount of markers used in the study was based on cost and time trade-offs, calculated probability of identity (PI) (Waits *et al.* 2001), the relative success in PCR amplification, and the interest in developing a method affordable to many researchers. Probability of identity is defined as the probability of obtaining identical genotypes given certain allele frequencies (Waits *et al.* 2001). Specifically the probability of identity of full siblings (sib), which is the probability that two siblings would share identical loci; a more conservative probability estimate. With the nine loci used in this study the probability of identity (sib) was 6.87×10^{-4} based on previously observed frequencies in past studies (Burg 1996; Davis *et al.* 2002; Hayes *et al.* 2006).

PCR reactions were performed in 25μL volumes consisting of 2-3 μL of sample gDNA, either 15 μL of GoTaq® Colorless Master Mix *(pH 8.5, 400μM dATP, dGTP, dCTP, dTTP and 3mM MgCl2)* (Promega, Wisconsin, USA) or 15 μL of KAPA2G Robust PCR Kits *[5X KAPA2G Buffer A, 5X KAPA2G Buffer B, 5X KAPA2G GC Buffer (all with Mg2+ at a 1X conc. of 1.5 mM) , 5X KAPA Enhancer 1 and extra MgCl2 (25 mM)]* (Kapa Biosystems, Massachusetts, USA), 0.5-1 μL of forward and reverse primers, and 6-7 μL of PCR grade water. The conditions of the PCR reactions varied among different loci depending on amplification success (Table 1). All PCRs were performed with a positive control of harbor seal skin blubber biopsies obtained from

Harriett Huber, NOAA National Marine Mammal Lab, Seattle, WA. While these samples did not have known reference genotypes they were advantageous as a positive control through the nature of the sample, as tissue samples provide ample quality gDNA for microsatellite analysis as compared to scat. This robust source of harbor seal DNA was an integral control to distinguish between issues pertaining to sample quality versus PCR-based issues. Samples that failed amplification when positive control amplified at locus signified that the sample was of poor quality. Samples were amplified for each locus and amplification was verified on a 1% TBE agarose gel.

After successful amplification, samples were purified for nucleotide sequencing using G-75 Sephadex columns and dried in a 96-well plate. Gel electrophoresis bands were qualitatively analyzed for concentration dilutions of 1:1, 1:5, or 1:25 to be re-suspended in 15 µL of a 1:20 dilution of Liz-500 Applied Biosystems (ABI) size standard in formamide. Amplified fragments were analyzed on an ABI Prism 3130XL Genetic Analyzer, and electropherograms were visually analyzed through ABI PeakScanner software to score alleles at each locus. Deviations from Hardy-Weinberg equilibrium and allele frequencies were determined using the program CERVUS; p-values were tested with Bonferroni correction (Kalinowski *et al.* 2007).

Sex Determination

To determine the sex of each sample, I used the qPCR assay from Matejusová *et al.* (2013) but only included controls of known samples and not the additional housekeeping gene (*CytB*). I ran all samples with positive controls of known male and female scat samples acquired from captive harbor seals at Vancouver Aquarium in Vancouver, BC and Point Defiance Zoo & Aquarium, Tacoma, WA. With known male and female scat samples I was able to confidently assign gender to field samples. The qPCR assay utilizes a Taqman qPCR assay that targets the homologs of zinc finger protein on the X and Y chromosomes (ZFX and ZFY) specific for harbor seals. The qPCR reaction consisted of 20 µL volumes of 1 µL of gDNA, 1 µL of TaqMan probe, 10 µL of ABI Taqman gene expression master mix, and 8 µL of PCR grade water (for primer sequences see Matejusová *et al.* 2013). Cycling conditions consisted of one holding cycle (50°C for 2 min, 95°C for 10 min) followed by 60 cycles of denaturation and annealing/extension (95°C for 15 sec, 60°C for 1 min). With these

known samples I confirmed sex determination through at least 4 consistent runs of Ct (cycle threshold) values. If there was ambiguity with these runs, I ran samples until I had at least 4 straight consistent results. With these consistent minimums, expecting that scat samples would be of lower quality DNA, I did not accept runs with Ct values over 40, consistent with Matejusová *et al.* (2013). However, I modified the original protocol by confirming male samples through positive amplification of ZFX and ZFY, as either present or absent, and confirmation by only accepting Ct values ≤40. Female samples were confirmed similarly through positive amplification of ZFX and negative amplification of ZFY and confirmation by only accepting Ct values ≤40 for ZFX. In addition to demonstrating the possibility of using scat for examining sex-specific ecological patterns, sex determination was also used as an additional control for individual identification. A workflow chart has been included to highlight the major methodologies included in this study (Figure 2).

Estimating Genotyping Error

I used a random number generator to identify 20% of the total samples size for re-amplification and repeated genotyping. This is a modification of the full multi-tube approach suggested by Taberlet *et al.* (1996), which advises at least seven re-runs per sample. A multi-tubes approach can be costly and a less lab-intensive statistical approach can also provide a robust estimation (McKelvey & Schwartz 2004; Schwartz *et al.* 2006). By only repeating a proportion of the samples (20% of total samples size), as suggested in a variety of reviews (Hoffman & Amos 2005; Pompanon *et al.* 2005), I was able to apply the re-run samples to computer programs that estimate genotyping error. Specifically, I used PEDANT, which applies a maximum likelihood estimation of allelic dropout (ADO) and false allele (FA) rates when there is an absence of reference data (typically with unknown individuals or in non-invasive genetic sampling) (Johnson & Haydon 2007).

Finding Matches through Virtual Genetic Tagging – A Probability Model

Generally, there are two objectives that researchers attempt to accomplish for genetically tracking individuals. One objective is to determine how likely an individual will be re-sampled. This question can be investigated by developing optimal sampling schemes to ensure, based on probabilities, that a study will obtain enough samples in the field to re-sample individuals. However, in genetic studies, researchers must also address

genotyping error, as it can lead to differences between genotypes of two distinct samples from the same individual. The second objective is therefore to determine whether or not the genotyping error rate observed in a study will inhibit the ability to identify those re-samples. One way to address this second objective is to determine, through re-run samples, where to assign a threshold in allelic differences for individual identification. While these objectives have previously been separated (determining re-samples with error and designing optimal sampling schemes for re-sampling individuals) they are inextricably linked when it comes to genetically tracking individuals.

Therefore, a Python-based computational probability model was created to effectively address the following main objectives: 1) simulate sampling schemes from virtual populations, 2) determine re-samples of individuals through allelic pairwise comparisons, and 3) optimize sampling schemes for future project development. The program consisted of three main scripts BEANBAG.py, WISEPAIR.py, and OPTIMAGIC.py (Figure 3). The BEANBAG.py script was specifically designed to build virtual individual genotypes of a population to be used in simulated sampling. This design was based on user-supplied criteria such as number of individuals in the population, number of loci, and allelic frequencies. In addition, this script incorporated genotyping error rates during sampling. The second script, WISEPAIR.py, was created to determine the number of re-samples within a specified data set (real or virtual) through allelic pairwise comparisons. WISEPAIR.py determined the number of re-samples within a virtual data set, determines the number of re-samples within an actual data using specified threshold simulations, estimates the number of errors for re-samples, and determines whether re-samples can be distinguished from non-re-samples. The final script, OTPIMAGIC.py, utilized outputs from both BEANBAG.py and WISEPAIR.py to develop optimal sampling designs for individual based studies. The following paragraphs explain the processes of each script:

BEANBAG.py

BEANBAG.py created a population with simulated genotypes followed by running a virtual sampling season on the population. It accepted a JSON file that contained number of loci (L), number of alleles (A), and allelic frequencies for respective alleles (AHz). From this JSON file it created a simulated population for

user specified number of individuals. This virtual population was used to construct genotypes for each individual using the provided AHz and a Pythonic implementation of the Mersenne Twister, a pseudo-random number generator (Matsumoto & Nishimura 1998). For each L the following processes began: (1) an A was randomly drawn, (2) its AHz was compared to a continuously randomized probability value (CRPV) from 0-1, (3) a particular A was assigned to an L when AHz is ≥ the probability value, (4) these three steps were then repeated for all loci for each individual until the virtual population was completely built. From this virtual population the script simulated a sampling season with user provided criteria, such as number of bouts and samples per bout. For each bout, the samples were pseudo-randomly chosen, without replacement, from the available individuals until the number of samples for that bout is met. The population list was refreshed for each bout.

To accurately address genetic sampling, the model incorporated simulations of allelic dropout and false allele error rates for the genotypes sampled. As described in the section estimating genotyping error, the model incorporated ADO and FA into the genotype for each individual using the PEDANT software suite (Johnson & Haydon 2007). These data were converted into JSON format and used to simulate genotyping error. PEDANT per-allele error rates were compared to a CRPV from 0-1. If the error rate was ≥ CRPV, then an error occurred for that allele; FA generated first, followed by ADO. It is important to note that the script places an "unknown allele" in for FA as it cannot determine what allele would actually be substituted, unlike a false allele in a real data set. For matching purposes the FA was treated as another allele and not ignored when matching genotypes. While this model accepted a virtual determination of error rates, it can incorporate previously determined rates by the user. Following these steps the sampling season is saved as a comma-separated variable (.csv) format. This standard output was used in the WISEPAIR.py scoring algorithm. The BEANBAG.py script was created for implementation and simulation of virtual populations and sampling needed when no data are available or included in iterative runs of the WISEPAIR.py script to determine threshold values for determining re-samples.

WISEPAIR.py

The second script was the WISEPAIR.py script, which either imported the standard output of BEANBAG.py or user-supplied data in .csv format. From these imports a full list of all pairwise comparisons for every sample was assembled. The pairwise list was run through a scoring function which compared the genotypes of each pair and returned a similarity score. Initially a raw similarity score (RSS) was determined, which is the sum of allelic differences of each pairwise comparisons where a lower score indicated higher similarity. A corrected similarity score (CSS[ ]) was then computed to account for variable number of loci being included (as some samples had missing data for certain loci) in the scoring of each pair (CSS = RSS/[# of loci used]). Each CSS was normalized (NCSS) by subtracting the overall CSS mean then dividing by the difference of the maximum CSS and minimum CSS:

$$NCSS = \frac{\left[CSS - \overline{CSS}\right]}{CSS_{max} - CSS_{min}}$$

When analyzing simulated data from BEANBAG.py, a "virtsim" ID code was included. This code allowed for error-free identification of individuals, even if ADO or FA have introduced discrepancies between identical genotypes. Using these IDs, WISEPAIR.py built a re-sampled threshold range for NCSS. The thresholds are established by using, minimum NCSS (with 95%CI), and maximum NCSS (with 95% CI), for the unpaired and re-sampled comparisons respectively. These ranges are applied later to real-world data sets to identify re-sampled individuals. The simulated NCSS were plotted onto histograms for visual inspection of the frequency distribution of re-sampled individuals and distinct, newly sampled individuals. The WISEPAIR.py and BEANBAG.py scripts were used for both the simulations in the following script and to determine the re-sample thresholds for this project's data set.

OPTIMAGIC.py

The third and final script used in the program was an optimization script. This script was developed to effectively optimize sampling schemes by iteratively running BEANBAG.py and WISEPAIR.py. The

possible variables included: number of bouts, samples per bout, counts of re-sampled individuals and count of times an individual is re-sampled over a season. Given all the specified variables, OPTIMAGIC.py performed simulations of all possible combinations of values or ranges using the previous scripts. BEANBAG.py and WISEPAIR.py iterated each scheme and determined a number of re-samples and non-re-samples, using the threshold model. Following these scheme simulations, all scoring data were parsed and re-sampled individuals were counted. These data were stored in two possible files. If the simulation met the specified re-sampled minimum and the mean number of times an individual is re-sampled then data for that sampling scheme were saved within the acceptable sampling file. If either of the criteria were not met for the simulations, then the sample scheme failed and was placed in the unacceptable sampling file. These data were then used to determine the best sample scheme for a given range of criteria.

Incorporating data into scripts through Cowichan Bay data

I used all three scripts to determine the number of individuals re-sampled within my data set. BEANBAG.py and WISEPAIR.py were used to produce a threshold "score" (refer to respective script methodologies) with which I could compare samples to the actual data set and subsequent simulations in OPTIMAGIC.py. For the WISEPAIR.py script I used error rates determined in PEDANT and calculated allele frequencies from my data. In order to effectively and confidently identify re-sampled individuals, my data set was compared (through thresholds from WISEPAIR.py) to simulated schemes under different conditions. These conditional simulations in OPTIMAGIC.py included a population based on my data-observed allele frequencies, number of alleles, and estimated error rates. OPTIMAGIC.py was used as a means to iteratively run BEANBAG.py and WISEPAIR.py for comparison purposes to my data set. However, these simulations were placed within sampling designs of either all re-sampled individuals or no re-sampled individuals. The all re-sampled individuals simulation included a population of 1,000 with sample limit 5,000 and a bout limit of 5; the no re-sampled individuals simulation included a population of 5,000 with sample limit 5,000 and a bout limit of 5. This excessive population size and extreme sampling scheme helped delineate the threshold value for my data set's pairwise allelic comparison. In conjunction with these extreme criteria, simulations that

incorporated more realistic parameters that matched Cowichan Bay were used with the same pipeline (1000 iterations of population 100 virtual individuals, 150 sample size, and 5 bouts). This massive iteration simulation was averaged from corrected threshold values for each iteration and compiled to determine threshold values for identifying isolate re-sampled individuals on my data set.

Optimizing for future projects using OPTIMAGIC.py

The final simulations determined the best sampling scheme for future individual-based genetic tracking studies at haul-out sites, such as Cowichan Bay. From a population of 100 individuals at Cowichan Bay (Olesiuk 2009), the OPTIMAGIC.py script was run to fit parameters that would include a high-frequency sampling effort (20-30 scats at each site during 20-25 bouts). This high-frequency simulation was used based on the assumption that researchers would want to re-sample individuals more frequently (at least 4-6 times per individual) than in my study. Due to permit restrictions for this study, I was unable to use a high frequency such as the one in this simulation. In addition to the high-frequency sampling parameters, I used a population of 100 individuals with using an estimate that a random 50% of individuals are absent from the haul out at any given time. Therefore a random 50 individuals are sampled during each bout; with replacement. While harbor seals can be extremely variable in their haul-out patterns based on life history factors (Brown & Mate 1983; Yochem *et al.* 1987; Huber *et al.* 2001) and can be locally variable (Thompson 1989), a modest estimate of 50% of seals hauled out is consistent with the previously observed estimates of harbor seal behavior. Using this scenario, the minimum number of individuals that would be re-sampled was 13/100, and these individuals would be sampled a minimum of 4 times or more. In addition, each scheme was iteratively run three times to give minimal stability to the output.

Results

Genotyping Individuals and Sex Determination

From the 46 scat samples collected I successfully genotyped 32 samples (~70% success rate) through at least seven of the nine loci used. Positive amplification varied among loci from 63% to 93%, with a mean

of 79%±SD0.11% for all samples (Table 2). Samples that either had too little available DNA from extracts (≤ 5 ng/μL) or more than 2 missing loci from failed PCR reactions were removed from the final data set. From these 32 samples I analyzed loci for number of alleles, allelic richness, expected and observed heterozygosity, % successful amplification, and observed base-pair lengths (Table 2). All loci were polymorphic within the data set, however two loci (*SGPv10* and *M11*) had moderate observed polymorphism with only 3 and 4 alleles respectively. Mean heterozygosity for the entire dataset was 0.76±SD0.19.

Loci did not deviate significantly from Hardy-Weinberg equilibrium, except *Pv11,* which had a heterozygote excess in observed heterozygosity as compared to expected (Table 2). With the allele frequencies observed in this study, I calculated the total observed probability of identity (sib) for all loci in this study as $2.78*10^{-2}$, which indicates that about 1 in every 36 full siblings are expected to share, by chance, an identical genotype. In addition, the probability of identity was $6.06*10^{-10}$ for non-sibling probability (Table 2).

For sex determination, I identified 11 female samples and 30 male samples. Five samples failed consistently to amplify and were not used for this study. The proportion of positive amplifications was 89%, not including the consistent amplification of all control scat samples of known males and females. Of the 41 positive samples, only the 32 samples that were successfully genotyped were used as a complete genetic tag including gender. However, the observed sex ratio of the haul-out during the sampling period should still be considered as roughly 3 males to a single female at Cowichan Bay.

Estimated Genotyping Error

Using the re-genotyped samples through PEDANT to estimate error rates I determined false alleles and allelic dropout rates for each locus (Table 2). The rates were not homogenous across loci and the loci with more alleles were the most informative due to PEDANT's ability to actually determine the error rates when more alleles were present. With the variety of rates per locus (ADO_{min}=0.00, ADO_{max}=0.21, FA_{min}=0.00, FA_{max}=0.25) the overall rates of mean allelic dropout per genotype across all loci were 6%±SD7% and false alleles across all loci were 12%±SD9%. The locus that was most problematic was *Lc26* which had the highest estimate error rates for both ADO and FA. This result could be attributed to the high FA rate as this introduced

more observed alleles into the pairwise comparisons. During the re-runs of FA, there were FA in all 6 repeated samples, with at least 2 having multiple false alleles (Appendix). While these false alleles this would not affect the FA rate as PEDANT does estimate this value on the whole, it may affect how simulations handle FA rates.

Individual Identification via Matching Thresholds

Simulation of non-re-sampled individuals resulted in a corrected threshold value of -0.353 with normal distribution (Figure 4). With all re-sampled individuals there was a significant tail at the threshold value of -0.412, with only a small proportion of samples reaching that limit (Figure 5). These two simulations were the basis for the threshold determination for Figure 6, which is applied to the sample data from my study. Based on these simulations of all re-sampled and not re-sampled individuals the threshold value identified 11 individuals that were re-sampled at least 1 time (Figure 6). Even with the extreme simulation parameters (all re-sample and no re-samples) and large population/sample size, simulations to determine threshold may need to incorporate more conservative estimates, to avoid type II error. An advantage to my small data set is being able to identify pairwise comparisons without the use of the scripts. Using this method instead of WISEPAIR.py and OTPIMAGIC shows that the 11 re-sampled individuals are isolate individuals. However, it is important to note that manual pairwise comparisons cannot incorporate genotype error, but can determine the number of allelic differences between samples.

Simulations included 1,000 iterations of a sampling design with a population size of 100 virtual individuals, 150 DNA samples, and 5 bouts. Through the model statistics described in the methods section, the threshold range was -0.542 in the lower bounds and -0.173 in the upper bounds. Examples of the simulations and their iteration patterns that built this threshold range are depicted in Figure 7. From these iterations the threshold value for my data set was the lower bound of the simulations, -0.173. This threshold value included two sets that were identified as a two recaptures (Pv14-28/Pv14-43 and Pv14-31/Pv14-33) (Figure 8). Based on this more conservative simulation, my study was able to match two sets of samples, to identify a recapture of two individuals.

OPTIMAGIC.py Results

Due to the limited number of individuals that were re-sampled, it was informative to determine the optimum sampling design for my system. Based on the parameters for an optimal sampling scheme (100 individuals with high frequency sampling of 20-30 samples per bout per 20-25 bouts), there were 5 different optimal schemes that would work in my system. The optimum schemes ranged from a minimum sample size of 690 samples over 23 bouts (30 samples per bout) to a maximum sample size of 750 samples over 25 bouts (30 samples per bout) (Figure 9). Optima visualized by bout number are represented in Figure 10. For the minimum optimum scheme, there were a total of 26 individuals that were re-sampled, with 13 that were re-sampled at least 4 times (mean count of re-samples per individual=5.66). For the maximum sample size, there were 34.67 re-sampled individuals with at least 23 individuals re-sampled a minimum of 8 times (mean count of re-samples per individual=5.81). These fluctuations of optimal sampling schemes were dictated by the range of bouts used (20-25) and the number of samples that could be taken per bout (20-30). There are 169 optimal schemes from the total data set that met the criteria to for count of re-samples per individual but did not meet the number of total individuals that meet that standard (13); these are yellow circle data points in Figure 9.

Discussion

While non-invasive genetic tracking has been a promising technique for researchers in wildlife science, the specific challenges and lack of empirical evidence leaves opportunities for methodological advancement (Beja-Pereira *et al.* 2009). With increased availability of technologies and the need for a well-planned experimental design (Schwartz & Monfort 2008, p 240; Hoban 2014), an empirical study examining the advantages and disadvantages of individual tracking in the wild is pertinent for the progression of this research. This is the first study to address the methodological considerations to non-invasive genetic tracking of harbor seals and develop an experimental design software specific for genetic tracking for individual-based studies in any taxa.

Results from this study show success in determining gender and identifying individuals through DNA sourced from scat samples. With considerable success in positive amplification (Table 2) as well as 89% success

in sex determination, my study shows a promising technique in our ability to obtain a reliable source of DNA through non-invasive means in harbor seals; especially as a technique that can be applied to species with site fidelity (haul-outs, wintering grounds, breeding grounds, etc.). The success in both microsatellite positive amplification (79%) and sex determination (89%) is consistent or higher than much of the literature regarding pinniped scats. Reed *et al.* (1997) observed 85% positive PCR results for harbor seal scats, however this value is only based in positive amplification for at least one microsatellite; for all microsatellites (5 total loci), they observed only 19.1% positive results. In ringed seals, using shed skin as a non-invasive sample, Swanson *et al.* (2006) observed 72% positive amplification in 6 microsatellite loci. With nine microsatellites used in this study, my positive amplification rate shows a highly reliable method to obtain quality DNA from non-invasive sources. With regard to sex determination, my results are consistent with Matejusová *et al.* (2013) as they observed 90% success with gray and harbor seal sex identification. Other examples of using *ZFX/ZFY* in pinnipeds [Crabeater (*Lobodon carcinophaga*), Ross (*Ommatophoca rossii*), and Weddell (*Leptonychotes weddellii*) seals] showed 80% success rate across species, however samples were from skin biopsies; a more invasive sampling methodology. One study using scat in harbor seals through an *SRY* gene specific sex determination observed only 44.5% successful identification (London 2006), supporting the effectiveness of a qPCR *ZFX/ZFY*-based assay. With the ranges of positive amplification success rates and variable number of markers, it is imperative that future studies adhere to a specific sampling and extraction method to ensure quality target DNA. In my study, swabbing proved to be quite successful in amplification success for both individual identification and sex determination.

Pv11 was the only locus to deviate from Hardy-Weinberg equilibrium. This deviation may be attributed to small sample size/population size, as there were no errors detected with this locus (Table 2). If there was consistent deviations across loci that would indicate population substructure or some form of relatedness among samples (Allendorf & Luikart 2007). It would be advantageous in future studies to consistently test this locus to ensure that there is no genotyping error associated with this result. The $PI_{(sibs)}$ observed in this study was reasonable with reference to harbor seal mating strategies. While there are little data on the mating systems of harbor seals, there is some genetic evidence of levels of polygamy in harbor seals (Hayes *et al.* 2006).

Importantly, $PI_{(non\text{-}sibs)}$ was well below ($6.06*10^{-10}$) any measure to ensure isolate individuals do not share similar genotypes, which may indicate that $PI_{(sibs)}$ is over conservative for my study. While $PI_{(sibs)}$ may be an advantage in some systems, the discrepancies due to a dichotomy between my observed $PI_{(non\text{-}sibs)}$ and $PI_{(sibs)}$ may be a result of the number of samples in this study. Conversely, this discrepancy indicates that while $PI_{(sibs)}$ may be high, my observed $PI_{(non\text{-}sibs)}$ can be a confident measure of identity.

The sex-determination assay proved valuable with even in the most degraded fecal samples (<5 ng/μL of gDNA). With the potential pitfalls of sample collection in non-invasive samples, such as DNA degradation and sample preservation, qPCR provides an advantageous tool that is a more sensitive and precise assay as compared to more traditional molecular assays (PCR amplification of sex – linked *ZFX/ZFY* and *SRY* genes, as shown in Shaw *et al.* (2003) and Reed *et al.* (1997) respectively). My study supports Matejusová *et al.* (2013) assay's effectiveness in their sex-determination method using DNA from scat. However, scat is not the only potential source of DNA at harbor seal haul-out sites. As a mammal, harbor seals also leave hair samples which can be a means for individual tracking if an effective methodology is applied (DeYoung & Honeycutt 2005). Future studies could investigate the use of hair snares at haul-out sites as another indirect means for identifying individuals, as performed in many terrestrial species and some aquatic mammals, such as otters (Beier *et al.* 2005; Depue & Ben-David 2007). The use of hairs may become more prevalent in non-invasive studies given the advancements in single-sample non-invasive hair-snare systems (Bremner-Harrison *et al.* 2006). In the case of dietary studies, scat would still be the most advantageous sample type as it can be used genetically for both individual identification and diet analysis (Thomas *et al.* 2014).

For the non-re-sampled and all re-sampled simulations, the combination of the Figures 4 and 5 into Figure 6 showed a clear representation of the binomial distribution expected when trying to match genotypes (McKelvey & Schwartz 2004). However, even with a binomial distribution of pairwise comparisons, the threshold value for determining matching individuals may not be conservative enough for individual identification. The number of allelic differences a researcher will allow to determine a match may dictate the threshold accepted in a study. It would be important in future studies, if cost is not prohibitive, to increase the number of loci used in the study. This will allow for strengthened confidence in identifying re-samples and

provide researchers with potentially more informative loci in genetic analyses. For my study, it was imperative not to commit a type II error, which would accept a matched individual when they are actually isolate individuals. Based on by-hand pairwise comparison, the raw number of allelic differences between simulated potential matched samples was greater than four, therefore it was important to run simulations that were more stringent. This larger number of allelic differences could be attributed to differences in frequencies of pairwise comparisons (Figures 4 and 5) that are vastly different in totals. The corrected threshold values are not relatable and when the simulations attempt to ID matches the threshold is too broad to correctly assign isolate individuals. Another explanation for failing to predict matching individuals is that while the simulation was based on observed allele frequencies, the conditions in which the population was built was based in an unrealistic sampling scheme (5000 samples over 5-sampling periods). The model itself was not developed to handle these types of schemes. To effectively address this inability to handle large populations and sampling schemes, it would be advantageous to build a null hypothesis for the program that would represent a method to detect differences among individuals. This alternative simulation may not incorporate extreme samples or population sizes, but rather extreme probability results within realistic sampling parameters. Specifically, this would be fitting an all re-sampling simulation and a no-resampling simulation yet match the number of pairwise comparisons as the real world data set. Another potential option would be to simulate pairwise comparisons with no genotyping error with large population sizes while also simulating large population sizes with observed genotyping error. Using the difference in threshold, under the assumption that even no genotyping error may mismatch by chance, this would provide a conservative threshold range for use on real world data. Future efforts will be in model development will be enhancing the script to appropriately handle large populations/sampling schemes for a null hypothesis.

Simulations that incorporated more stringent parameters and were run iteratively proved to be the most informative threshold for my data set (Figure 7). The simulations provided an acceptable number of allelic differences that would be an informative threshold. For individual identification, I successfully tracked two individuals within our data set with our method to determine individuals. The variety of different simulations used allowed for an accurate representation of the difference in threshold values. It would be

beneficial in future studies to use recapture rates as a means to extrapolate out recaptures based on number of samples. While this study highlights optimum sampling schemes, the recapture rates are based on simulated populations that have assumptions on sample success/failure rates and therefore cannot buffer these rates into simulations. The present simulations would benefit from an included recapture rate from empirical data, potentially through studies specifically addressing this assumption.

The two instances of re-sampled individual were not sufficient for tracking harbor seals in instances of parsing individual differences within a population. This finding was most likely a result of a small sample size within my data set. Given logistical constraints I was unable to increase the number of sampling bouts. Previous studies have noted the number of samples needed in non-invasive studies with some recommending 2.5-3 times the number of fecal samples as the number of animals expected to be sampled (Solberg *et al.* 2006; Marucco *et al.* 2011). This number for fecal samples is based on 20-30% of samples unable to be genotyped. With this argument, the number of samples needed to effectively track 100 harbor seals at Cowichan Bay would be 250-300 samples. However, suggested samples size is usually based on capture-recapture studies for population estimation where is not necessary to have a multiple recaptures per individual. This general estimation it is not consistent with the OPTIMAGIC.py output which recommended, at minimum, 690 samples to effectively track at least 13 individuals in a population of 100 seals at Cowichan Bay (assuming 50% are absent at any given sampling period). Much of the previous literature uses assumptions for the estimates that fail to incorporate the facets of sampling design for a successful non-invasive study, namely genotyping error which can affect a project's ability to match individual genotypes (Taberlet & Luikart 1999).

OPTIMAGIC.py provides a starting point for researchers to determine how many samples they would need to effectively track individuals in an individual-based studies. This model incorporates criteria such as genotyping error, number of re-samples, and number of times an individual will be re-sampled that are important parameters for projects attempting to genetically track individuals in the wild. The range of OPTIMAGIC.py optimal schemes (see Results), highlights the power of the model and the variety of schemes that can work for researchers in a given question. It is unclear whether any one of the 5 schemes would be the "best" option for my particular system. The fluctuations optima are related to the combinations of bouts and

sample sizes that dictate changes to whether or not it will meet an optimum. With the pseudo-random nature of some of the algorithms as stated in the methods, there will be events in the simulations that create fluctuations of optimal schemes. The advantage to OPTIMAGIC.py is that researchers can choose from the data set and re-run OPTIMAGIC.py iteratively under one scheme. This output could give better insights into the whether or not an individual scheme matches the researchers' guidelines. For example, in my study, the cost per sample was a concern due to logistics and therefore it may be advantageous to use the smallest sample size possible from the OPTIMAGIC.py results. With this research limitation the smallest samples size to ensure I could re-sample individuals effectively would be 690 samples over 23 bouts (30 samples per bout). It is important to note that 20-30 visits to a haul out site could be deemed invasive on harbor seals due to repeated harassment (Suryan & Harvey 1999). While typically genetic samples have been obtained through capturing an animal (tissue and blood), scat still serves as a minimally invasive option; even with disturbing a haul-out site. The advantage to OPTIMAGIC.py is its ability to use previous data to estimate sampling schemes *a priori* for potential future studies of individual tracking. The input parameters can be extensive for which researchers choose to use, including effective sampling population size, genotyping error rates, % present, number of individual samples and how many instances they are sampled.

There are some assumptions included in the pipeline described in simulation figures 4-10 that should be addressed in future studies. One is the assumptions that all samples are of the same quality (for scat, freshness based on time since defecation). There has been some work in the literature to determine the DNA degradation rates of scats in the field (Piggott 2005; Murphy *et al.* 2007; Brinkman *et al.* 2010). A recent study investigating fecal deposition rates and DNA degradation to optimize sampling scheme in Sonoran pronghorn (*Antilocapra Americana sonoriensis*) determined that a sampling interval of 4-7 days under summer conditions proved most advantageous (Woodruff *et al.* 2014). However these rates may be site- or species-specific and would be important for future studies with samples in marine environments to assess the percent of degradation affecting the number of samples needed. This assessment would allow OPTIMAGIC.py to appropriately buffer for these samples that may fail in the field. However, if not applicable in the OPTIMAGIC.py program, future studies should plan to buffer for these failed samples regardless. In addition, microsatellites have high

mutation rates (Ellegren 2000) and it may be of importance to include estimating these rates into the building of virtual genotypes. There are studies that have estimated mutation rates, starting from the simplest model of stepwise mutation model (Ohta & Kimura 1973) that uses the length of repeat units moves one unit (both expanding and contracting) to more recent models (Whittaker *et al.* 2003) that use likelihood based models. Another assumption is this study stated that a false allele would be treated as another allele. However, in real data sets, a false allele can range in base pair length depending on how many false alleles are present within a locus. For instance, locus *Lc26* was observed to have the highest false allele rate (Table 2) and in the current simulation this the false alleles present would be pooled together and represented as a single error instance. Through analysis of the re-run data set there are potentially differences in allele scores from 2 base pairs up to 16 base pairs, all incorporating six new alleles that may be deemed a false allele. However, the rate at which these new alleles are presented, or rather the likelihood that one false allele may be present is unknown. This assumption can overinflate the number of false alleles present and potentially hide matching genotypes in a data set or simulation.

Such as the aforementioned assumptions, there must be consideration when working with non-invasive samples in genotyping error and its effect on determining individuals. While my study addressed genotyping error, it is imperative that future studies continue to redefine the issues associated with genotyping error (Taberlet & Luikart 1999; Waits *et al.* 2001; Pompanon *et al.* 2005). For future studies, I stress the importance of a well-developed molecular control system (such as known control and a gender determination assay) and a modest number of repeat PCRs of samples. This will greatly reduce the potential for fluctuating errors among loci, samples, and future studies that intend to compare empirical data. It is also important to highlight the sampling methodology used for fecal sampling as this can drastically affect DNA quality for downstream analysis (Lampa *et al.* 2008; Rutledge *et al.* 2009). My method using a sterile swab to target sloughed epithelial cells improved my lab amplification success and quality of DNA, which can further mitigate the issues with genotyping error.

Non-invasive genetic tracking for individual based studies has shown some signs of traction in the literature, notably in amphibians (Ringler *et al.* 2014) and fish (Andreou *et al.* 2012), yet the application of these

tools has yet to be fully appreciated. While there are a multitude of questions that come from individual-level data, the species specific methodological considerations are imperative to a successful project. My study successfully highlighted laboratory and sampling design considerations for harbor seals that may be applicable to taxa in which researchers can reliable obtain genetic samples in high frequency. Specifically in species with some fidelity to a location (haul-outs, latrines, breeding grounds, etc.), the ability to apply this technique can be highly successful for genetic tracking. The complexities of tracking these species come from developing an adequate workflow to mitigate the many pitfalls associated with non-invasive genetics while securing a sampling scheme that aligns with researchers' project objectives. This study provided an alternative path to either a full re-run approach or a solely statistical approach to matching individuals. Based on cost and logistics, it is important that future studies identify the tradeoffs among differing methods and apply the most robust techniques and available tools to address matching genotyping and errors associated. Nevertheless, this hybrid approach allowed for minimal sacrifice in the available methods as it incorporated sample re-run, allelic pairwise comparisons, and probabilistic simulations to determine matching thresholds. This study effectively developed a computer program tool that researchers can use for projects in individual genetic tracking by optimizing sample size through incorporating effective sampling population size, genetic error rates, and non-invasive sampling (sampling with replacement). With the combined results of this study, researchers can expect to develop more robust data sets that capture differences among individuals while addressing logistical and financial concern that can lead to prohibitive research methodologies. As reiterated in much of literature (Palsbøll 1999; Taberlet & Luikart 1999; Valiere *et al.* 2007) a pilot study is critical to the success of these methods. Future studies can use the Python-based scheme to develop *a priori* a sampling design to conduct individual-based studies on any taxa. In addition, researchers can follow my lab procedures to genetically track individual harbor seals to study ecological traits, such as the diet of individuals.

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Tables and Figures

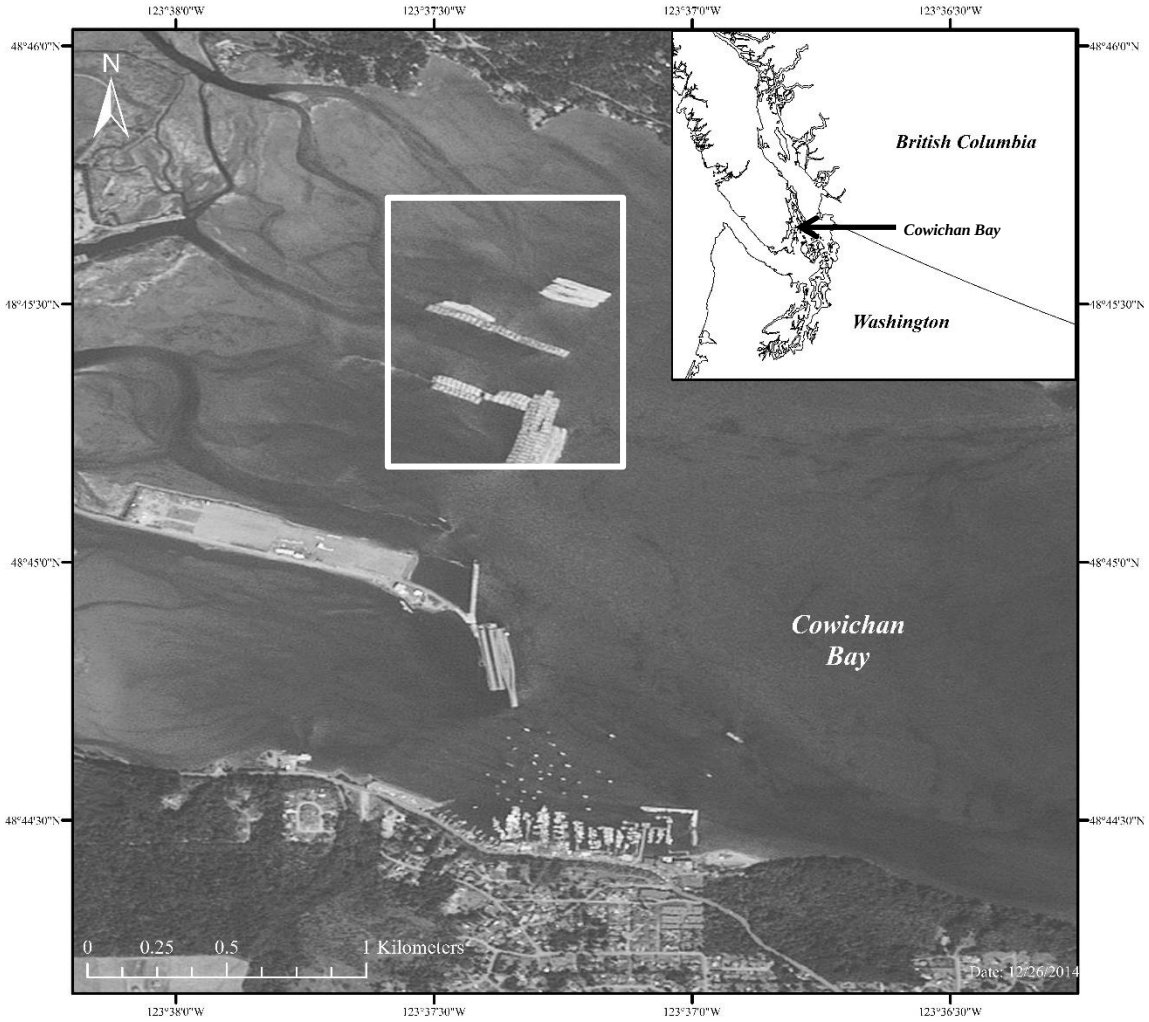

Figure 1. Cowichan Bay, the study site on Vancouver Island, BC. The log booms where samples were collected are located north of Cowichan Bay Marina, and are indicated with a highlighted rectangle in the figure

Locus	*A°*	*Conditions*	*Master Mix*	*Primer Source*
HI15	52	1 cycle initial of 94° (3 mins), 52° (1 min), 72°(1 min) ; 38 cycles of denaturation at 94° (1 min), annealing temperature (1 min), extension at 72° (1 min); Final extension at 72° (10 mins)	GoTaq®Colorless	Davis *et al.* (2002)
LW20	52	1 cycle initial of 94° (3 mins), 52° (1 min), 72°(1 min) ; 38 cycles of denaturation at 94° (1 min), annealing temperature , extension at 72° (1 min); Final extension at 72° (10 mins)	GoTaq®Colorless	Davis et al. (2002)
TBPv2	48/51	Initial of 94° (2 mins); 11 cycles of denaturation at 94° (1 min), lower annealing temperature (1 min) , extension at 72° (1 min); 27 cycles of denaturation at 94° (1 min), higher annealing temperature with 0.1° touchdown (1 min), extension at 72° (1 min); final extension at 72° (7 mins)	GoTaq®Colorless	Burg (1996)
M11	48/52	Initial of 94° (5 mins); 12 cycles of denaturation at 94° (1 min), lower annealing temperature (1 min), extension at 72° (1 min); 25 cycles of denaturation at 94° (1 min), higher annealing temperature (1 min); final extension at 72° (7 mins)	GoTaq®Colorless	Gemmell *et al.* (1997)
SGPv10	55	Initial of 94° (2 mins); 40 cycles of denaturation at 94° (15 sec), annealing temperature (15 sec), extension at 72° (15 sec); final extension at 72° (7:00 min)	KAPA2G Robust	Burg (1996)
Lc5	55	See above	KAPA2G Robust	Davis et al. (2002)
Lc26	59	See above	KAPA2G Robust	Davis et al. (2002)
Pv11	59	See above	KAPA2G Robust	Gemmell *et al.* (1997)
BG	59	See above	KAPA2G Robust	(Burg (1996))

Table 1. Loci of nine microsatellites used in this study. Each locus has its corresponding cycling conditions for PCR optimization before fragment analysis. Primer sequence sources are also listed.

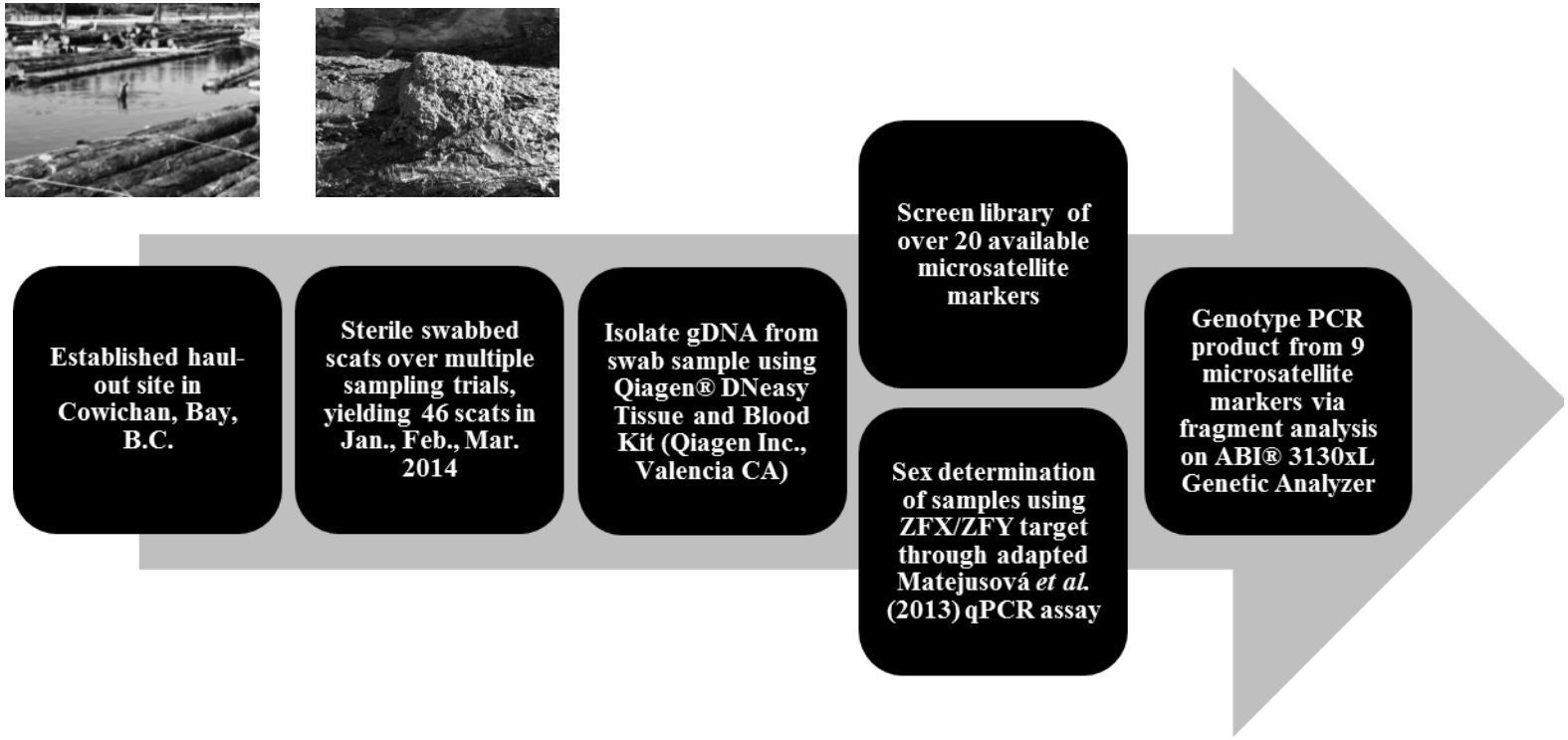

Figure 2. A workflow for non-invasive individual identification of harbor seals (Phoca vitulina) using scat swab samples. This methodology could be applied to other species, especially those that spend some time on land (semi-aquatic species). Multiple collection trips allow for genetic tagging of individuals

Locus	No. of Alleles	BP Range	% + PCR	H_{exp}	H_{obs}	$Prob_{(sib)}$	ADO per genotype rate	FA per genotype rate
HI15	9	119-139	93%	0.79	0.84	$1.24*10^{-1}$	0.05	0.11
Lc5	5	160-168	65%	0.65	0.50	$5.84*10^{-2}$	0.01	0.24
Lc26	9	305-327	83%	0.80	0.66	$2.15*10^{-2}$	0.21	0.25
M11	4	145-151	83%	0.69	0.88	$9.60*10^{-3}$	0.00	0.13
SGPv10	3	129-133	89%	0.48	0.47	$5.70*10^{-3}$	0.10	0.11
TBPv2	11	234-256	63%	0.86	0.81	$1.90*10^{-3}$	0.00	0.10
Pv11	8	154-168	80%	0.72	0.97*	$8.00*10^{-4}$	0.00	0.00
BG	7	283-310	76%	0.79	0.91	$3.00*10^{-4}$	0.10	0.00
Mean	***7***	*N/A*	**79%±0.11**	**0.72±0.12**	**0.76±0.19**	**$2.78*10^{-2}$†**	**0.06±0.07**	**0.12±0.09**

Table 2 Loci and their corresponding number of alleles, percentage of positive PCRs per locus (% + PCR), expected and observed heterozygosities, probability of identity for siblings, allelic dropout and false allele rates estimated from PEDANT. Mean values reported for respective parameters, as well as allelic richness for all nine loci. Mean values are ± standard deviation. Percentage of positive PCRs are from samples that successfully amplified in at least 7 of 9 loci. *Significant deviation ($p<0.05$) when Hardy-Weinberg equilibrium test was conducted; for locus Pv11, χ^2 (3, N=31) p=0.00 using CERVUS (Kalinowski et al. 2007). †Probability of identity (sib) is a total value, probability of identity (non-sib) total = $6.06*10^{-10}$.

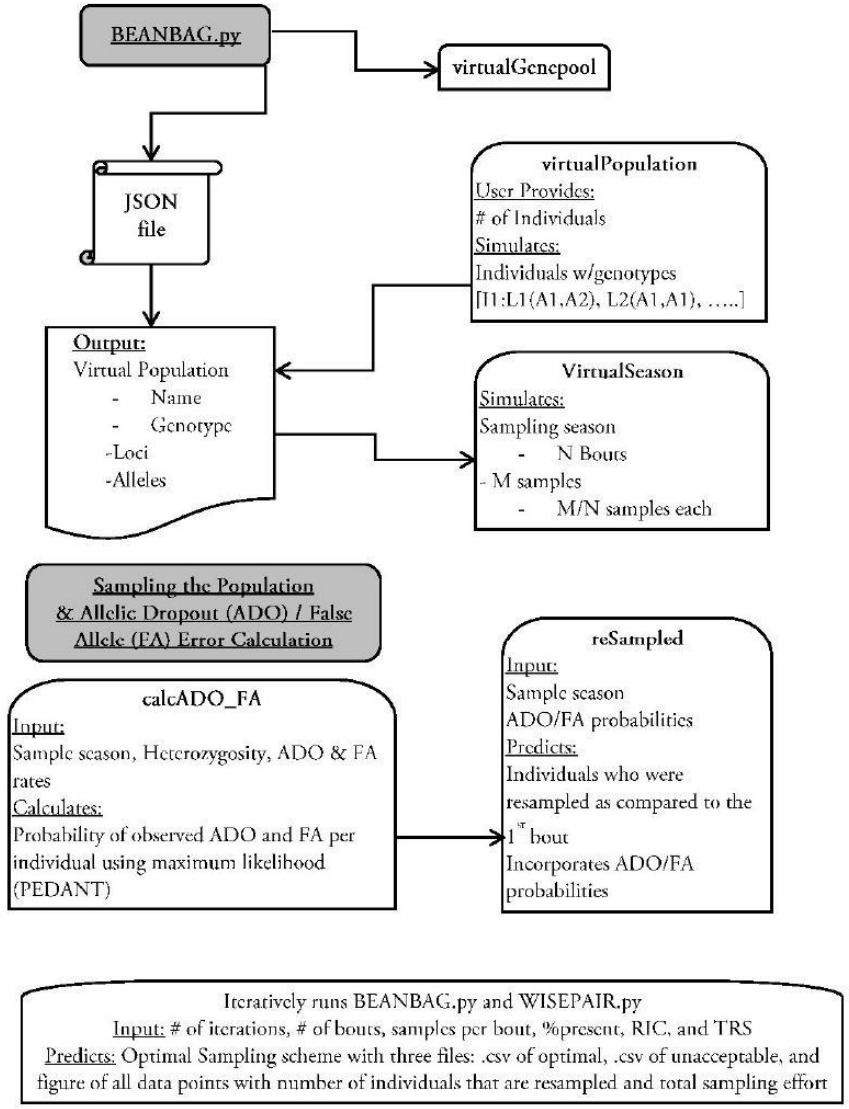

Figure 3. A workflow of a Python script to simulate a non-invasive sampling design with either input data from user supplied or virtual gene pool. Grey boxes highlight important scripts used in the pipeline. Virtual sampling incorporates allelic dropout and false allele rates through PEDANT. OPTIMAGIC.py can be run for determining optimal sampling schemes using BEANBAG.py and WISEPAIR.py iteratively.

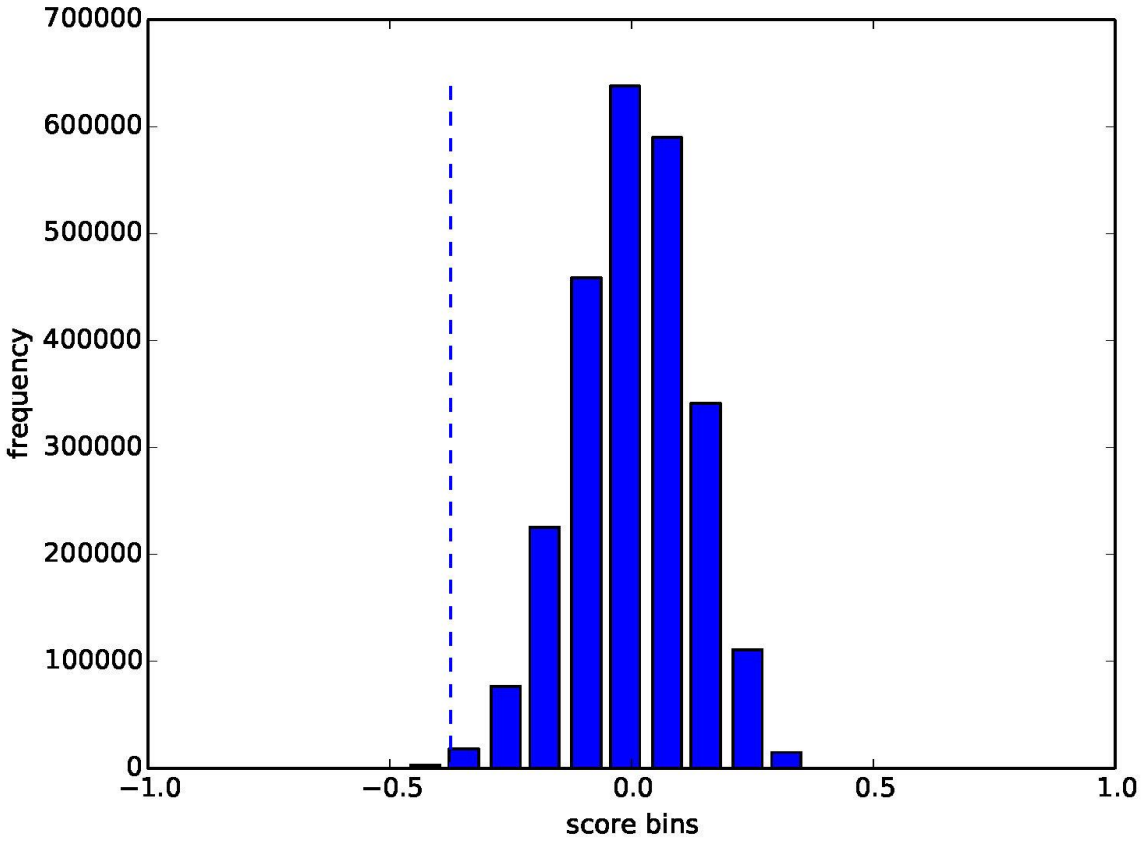

Figure 4. Histogram of WISEPAIR.py simulation of 5,000 individuals with 5,000 total samples over 5 sampling bouts. The blue bars show that or: none of the pairwise comparisons consisted of re-samples. The blue dotted line represents 95% confidence interval at -0.353. Frequency corresponds to pairwise comparison of individual genotypes that were binned into corresponding corrected scores. The virtual population was created based on this study's observed allelic frequencies and estimated error rates.

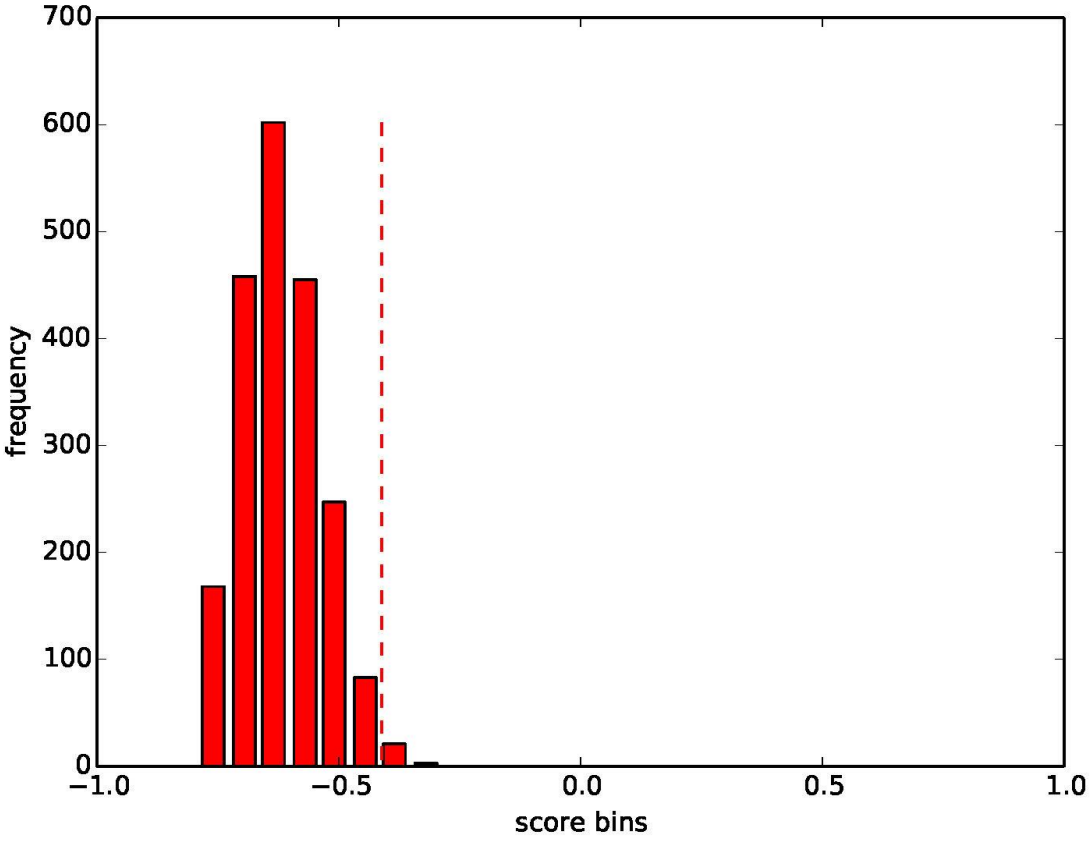

Figure 5. Histogram of WISEPAIR.py simulation of 1,000 individuals with 5,000 total samples over 5 sampling bouts. The red bars show that all pairwise comparisons consisted of all re-sampled individuals. The red dotted line represents 95% confidence interval at -0.412. The lower bound of the distribution shows the likelihood that those comparisons incorporate genotyping error into the simulations as they may be non-re-sampled genotypes. The virtual population was created based on this study's observed allelic frequencies and estimated error rates, with the exception that the simulation would include all re-samples.

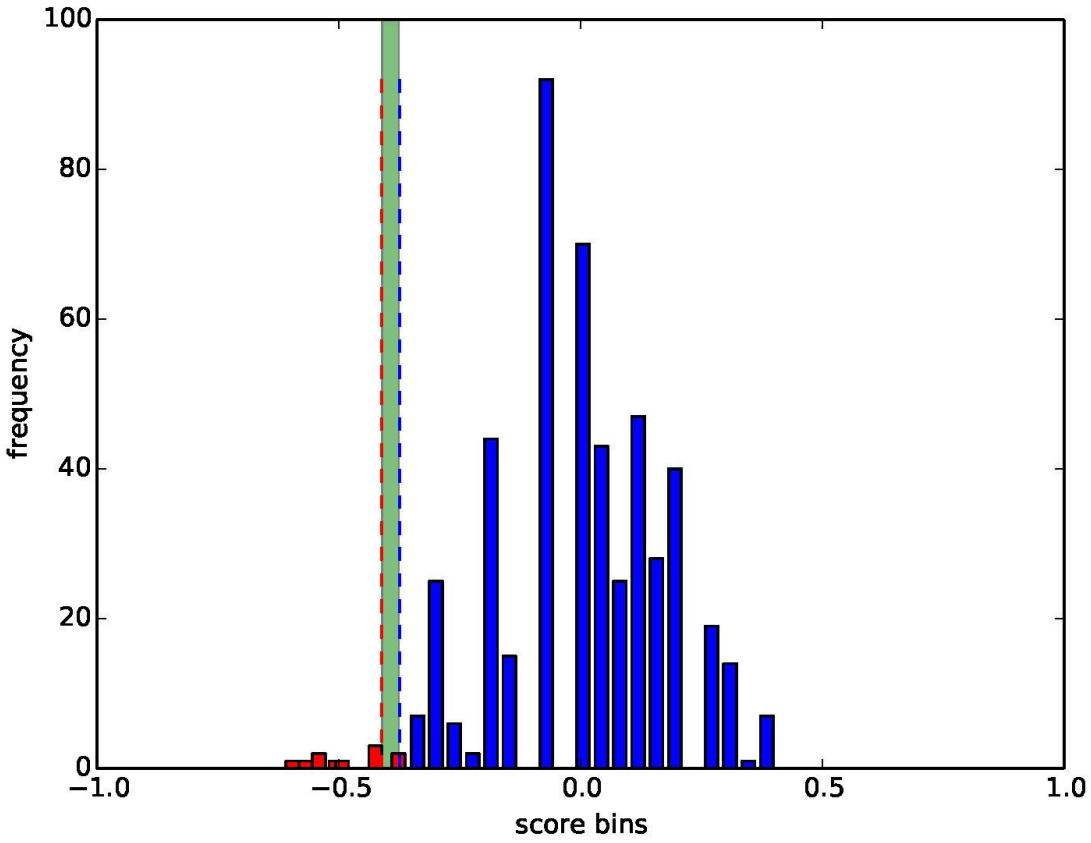

Figure 6. Histogram incorporating the upper and lower bound thresholds from simulations of Figure 4 and 5 to Cowichan Bay pairwise data set. The range of the threshold incorporated 11 individuals, shown in red that are re-sampled (some samples multiple times) under these conditions. The area between the two threshold bounds (shown in green) is interpreted as pairwise comparisons that have no ambiguity in assigning non-re-sampled comparisons from isolate, re-sampled comparisons.

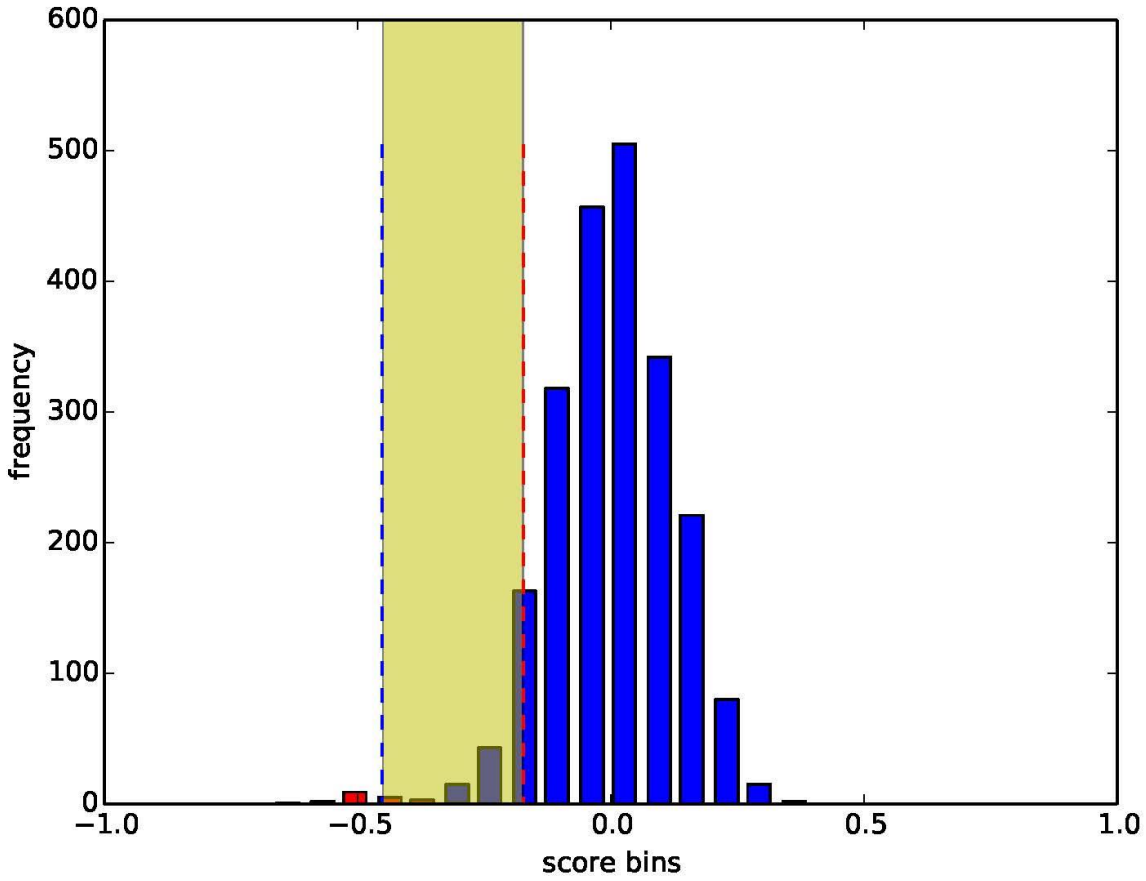

Figure 7. Example of one iteration from 1,000 simulations of a population of 100 individuals with 150 samples over 5 sampling bouts. Unlike what is depicted in Figure 6, the area between threshold bounds incorporates pairwise comparisons that have some ambiguity (overlapping 95% confidence intervals) that include possible matches or isolated individuals. In the case of my study, these are not included to be certain on individual genotyping calls.

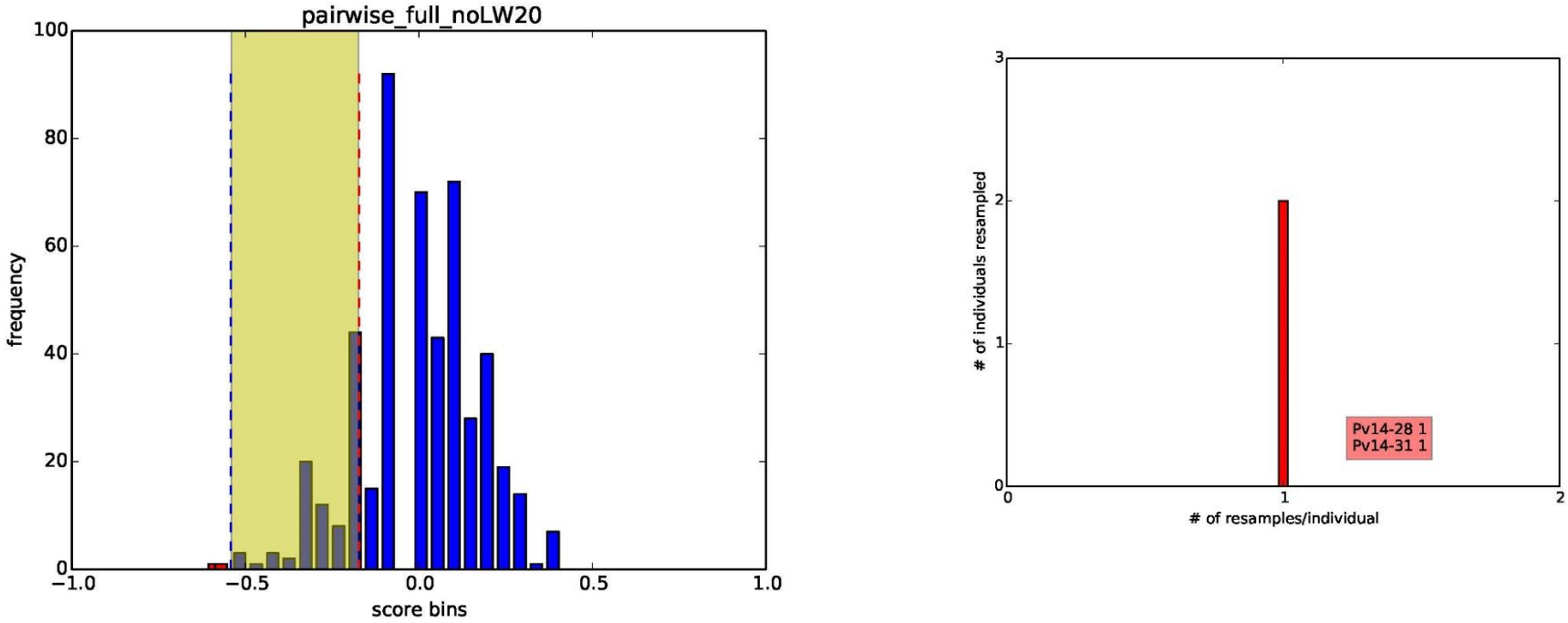

Figure 8. Based on 1,000 iterative simulations to determine threshold values for re-sampled individuals, the bounds of the corrected score were included in the histogram of pairwise. Based on the lower bound threshold, the simulations determined that there were two pairs of samples that were identified as re-sampled individuals (1) Pv14-28 and Pv14-43 and (2) Pv14-31 and Pv14-33, which are shown in the second graph of number of individual re-sampled

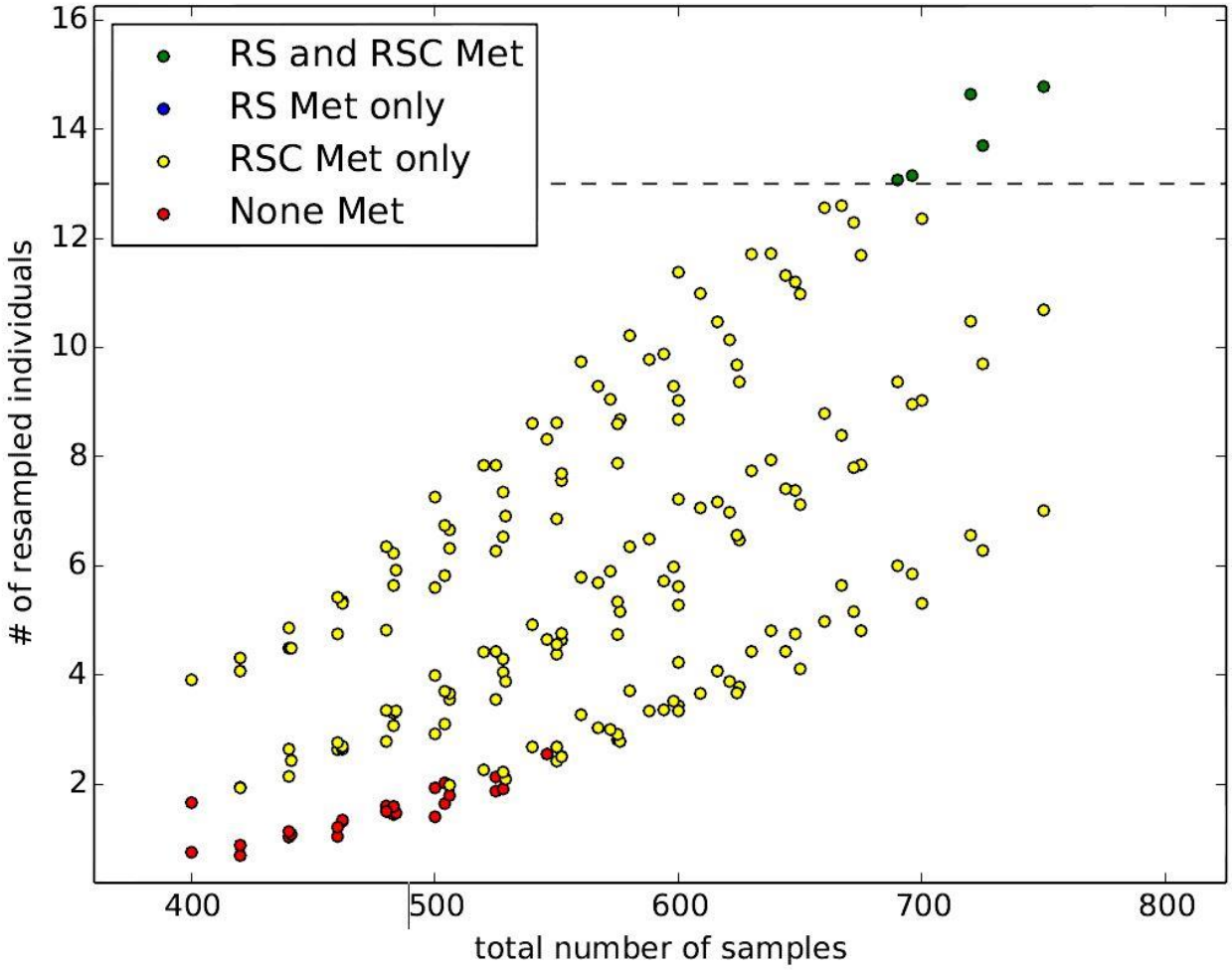

Figure 9. OPTIMAGIC.py optimal sampling schemes for a population of 100 individuals with a random 50% absent at any given bout. Criteria included a sampling effort of 20-30 scats for each visit for 20-25 bouts. Dotted line represents the minimum number of re-sampled individuals (13) sampled at least 4 times. Each scheme was iterated three times. Data points above dotted lines represent schemes that met both criteria of re-sampled counts (RSC) and number of individuals re-sampled (RS), in green. Yellow circles only met one of the criteria and red dots represent schemes that met none of the conditions. There were 5 schemes that met the input criteria that ranged in sample sizes of 690-750 total samples.

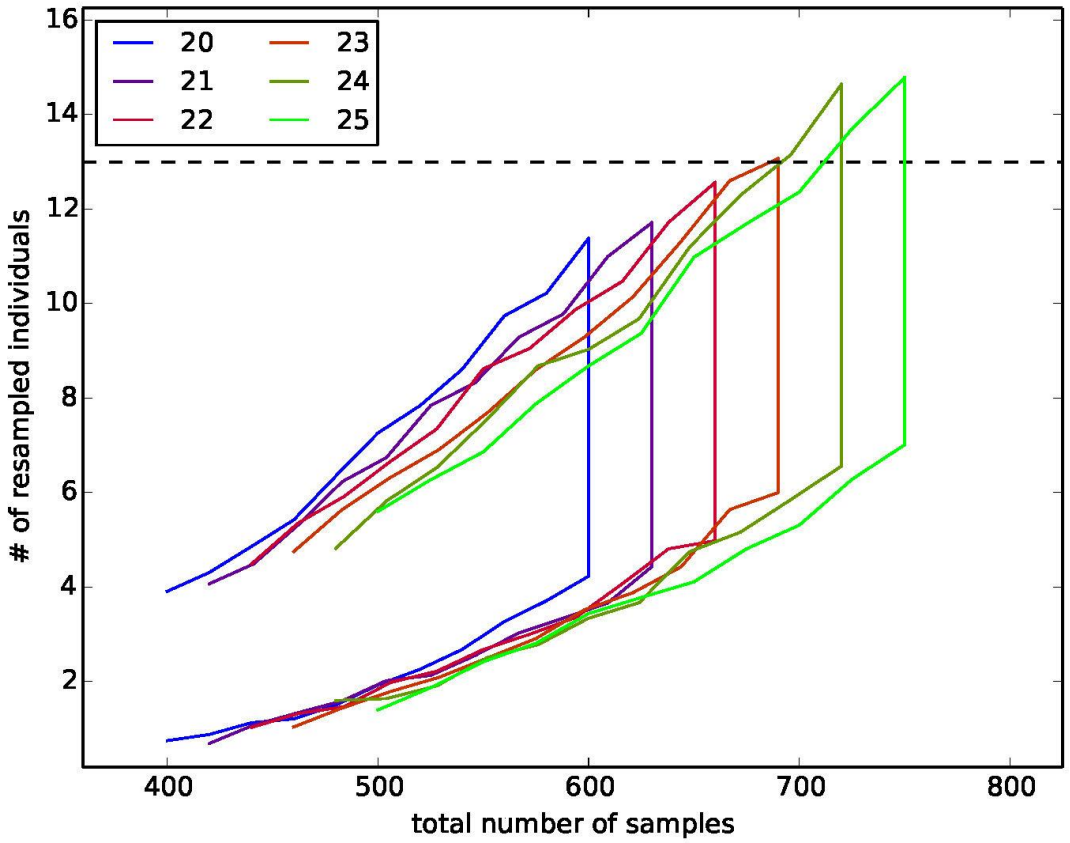

Figure 10. Trace of optima based on number of bouts. Criteria included a population of 50 individuals with a sampling effort of 20-30 scats for each visit for 20-25 bouts. Vertical lines at end of each line correspond to the maximum number of samples for the respective bout number. Figure 9 depicts the individual schemes. Legend matches line color to number of bouts.

Appendix

Sample	**LW20**	**LW20**	**HI15**	**HI15**	**Lc5**	**Lc5**	**Lc26**	**Lc26**	**M11**	**M11**	**SGPv10**	**SGPv10**	**TBPv2**	**TBPv2**	**Pv11**	**Pv11**	**BG**	**BG**
Pv14-01	122	130	122	130	164	164	313	313	149	149	131	131	247	247	0	0	305	305
Pv14-02	126	140	122	122	164	166	315	317	151	151	131	133	232	252	155	155	299	299
Pv14-03	128	134	122	142	0	0	307	315	145	147	131	133	252	252	160	162	284	305
Pv14-04	126	130	120	122	164	166	307	319	149	149	131	133	250	250	166	166	294	294
Pv14-05	124	124	130	142	164	164	307	315	145	149	131	133	0	0	160	160	0	0
Pv14-07	126	136	120	122	164	166	307	319	147	149	131	133	247	256	162	162	305	310
Pv14-08	134	140	120	122	164	164	307	307	149	151	133	133	240	240	156	162	294	299
Pv14-09	124	142	124	130	164	164	307	307	149	149	131	133	254	254	162	162	0	0
Pv14-10	0	0	120	128	166	166	307	317	149	149	131	133	250	250	155	155	284	299
Pv14-12	124	134	122	122	164	166	307	319	147	149	131	131	232	256	162	162	304	310
Pv14-13	116	116	120	122	164	164	307	317	147	149	131	133	244	252	160	160	299	310
Pv14-16	124	140	105	122	164	166	323	323	149	149	133	133	244	254	162	162	294	305
Pv14-17	134	140	124	124	164	166	307	317	149	149	133	133	244	252	160	168	284	299
Pv14-18	132	132	122	122	164	166	307	317	147	151	133	133	250	250	160	160	310	310
Pv14-19	132	134	122	142	0	0	307	315	147	151	133	133	244	254	162	162	294	305
Pv14-22	126	128	0	0	164	166	305	319	147	151	131	131	246	246	160	160	299	305
Pv14-23	124	126	122	122	0	0	315	327	147	147	131	131	240	240	162	162	305	310
Pv14-24	124	126	122	122	166	166	317	317	149	149	131	131	250	250	160	160	0	0
Pv14-25	128	130	130	130	164	166	307	307	0	0	131	133	0	0	160	160	299	305
Pv14-27	126	130	122	130	0	0	307	317	149	149	131	131	0	0	162	162	0	0
Pv14-28	130	132	122	122	166	168	317	321	147	149	131	131	0	0	162	162	299	310
Pv14-30	124	124	122	130	164	166	319	319	0	0	131	131	250	250	160	160	299	305
Pv14-31	124	126	122	130	166	168	307	315	145	151	131	131	250	250	162	162	299	310
Pv14-33	126	126	122	130	166	166	307	315	151	151	131	131	244	244	162	162	299	310
Pv14-34	130	132	122	122	0	0	321	321	149	149	133	133	0	0	0	0	310	310
Pv14-35	126	140	122	132	164	168	317	319	149	149	131	131	0	0	160	160	284	299
Pv14-38	124	126	124	124	0	0	323	323	147	147	129	131	250	250	160	160	305	305
Pv14-39	0	0	122	122	168	168	317	317	0	0	131	131	250	250	162	162	284	284
Pv14-43	0	0	132	132	168	168	317	321	149	149	131	131	234	250	160	160	299	310
Pv14-44	130	132	122	122	166	168	307	319	147	149	133	133	252	252	154	160	299	305
Pv14-45	124	126	122	130	0	0	317	317	147	159	131	131	250	250	160	166	294	294
Pv14-46	128	130	130	130	160	164	317	319	149	149	133	133	250	250	160	160	284	284

A1. *Microsatellite raw data for 9 loci used in analysis*

Sample	HI15	HI15	Lc5	Lc5	Lc26	Lc26	M11	M11	SGPv10	SGPv10	TBPv2	TBPv2	Pv11	Pv11	BG	BG
Pv14_01_1	123	129	162	164	313	313	147	149	129	131	246	248	0	0	299	305
Pv14_10_1	121	123	166	166	307	317	149	151	131	133	248	250	154	156	284	299
Pv14_12_1	121	123	164	166	307	319	147	149	131	131	254	256	160	162	305	310
Pv14_16_1	121	121	164	166	323	323	147	149	131	133	244	254	160	162	293	305
Pv14_23_1	121	123	0	0	315	327	145	147	131	131	238	240	160	162	305	310
Pv14_27_1	123	131	0	0	307	317	149	149	131	131	0	0	160	162	299	305
Pv14_01_2	123	129	164	166	315	317	0	0	129	131	248	250	154	156	299	305
Pv14_10_2	123	131	164	166	317	319	149	151	131	133	248	250	154	156	284	284
Pv14_12_2	121	123	164	166	307	319	147	149	131	133	246	256	160	162	305	310
Pv14_16_2	121	123	164	166	307	321	147	149	131	133	244	254	160	162	293	305
Pv14_23_2	121	123	164	166	313	315	147	151	129	131	240	254	160	162	0	0
Pv14_27_2	129	131	166	168	305	307	0	0	0	0	240	252	160	162	299	305

A2. *Microsatellite re-run raw data for 9 loci. Estimates for genotyping error rates determined using PEDANT software*

Sample	*Gender*
Pv14-01	M
Pv14-02	M
Pv14-03	M
Pv14-04	F
Pv14-05	M
Pv14-06	M
Pv14-07	F
Pv14-08	M
Pv14-09	M
Pv14-10	F
Pv14-11	N/A
Pv14-12	F
Pv14-13	M
Pv14-14	M
Pv14-15	F
Pv14-16	M
Pv14-17	M
Pv14-18	F
Pv14-19	M
Pv14-20	N/A
Pv14-21	N/A
Pv14-22	M
Pv14-23	M
Pv14-24	M
Pv14-25	M
Pv14-26	M
Pv14-27	M
Pv14-28	M
Pv14-29	M
Pv14-30	F
Pv14-31	F
Pv14-32	N/A
Pv14-33	F
Pv14-34	M
Pv14-35	F
Pv14-36	M
Pv14-37	M
Pv14-38	M
Pv14-39	F
Pv14-40	N/A
Pv14-41	M
Pv14-42	M
Pv14-43	M
Pv14-44	M
Pv14-45	M
Pv14-46	M

***A3**. Gender determination results for all samples. "M" represents male, "F' represents female, "N/A" unable to be determined through 4 repeat runs of qPCR assay.*